

Response of Old Skeletal Muscle to 8 Weeks of Electrical Stimulation (Should We Change the Conventional Electrical Stimulation Protocol for Cardiomyoplasty?)

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Abstract

We hypothesized that the conditioned muscles of elderly organisms have different responses to electrical stimulation than that of young adult organisms. One-year-old sheep and eight-year-old elderly sheep were used for this investigation.

Results. The latissimus dorsi muscle (LDM) of old sheep has less fatigue resistance than LDM of younger animals. In all animals, LDH-5 fractions decreased after eight weeks ES; LDH-1+2 fractions increased. After a two week delay, the data completely returned to baseline values in old adult animals. The percent area occupied by mitochondria in old sheep was less after ES than in younger animals. In all animals, the mitochondrial area increased after ES and reverted to baseline values after the delay. The number of nuclei and fibers considerably increased after ES.

Conclusions. Young skeletal muscle obtains more plasticity than adult muscle during ES. Elderly skeletal muscle does not convert to a fatigue resistant state as completely as adult skeletal muscle during a conventional eight week ES protocol. It is necessary to change and prolong the ES protocol for elderly patients.

Key words: ageing, dynamic cardiomyoplasty, electrical stimulation protocol, muscle, sheep.

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The discovery that after several weeks of low frequency electrical stimulation adult skeletal muscle is capable of undergoing adaptive changes and acquires increased fatigue resistance [9-11] has piqued interest in the possible use of transformed skeletal muscle for surgical treatment (cardiomyoplasty) of end-stage congestive heart disease [1, 6]. Despite remarkable symptomatic improvement in cardiomyoplasty patients, there is no concurrent objective hemodynamic improvement [2, 4, 6]. A loss in hemodynamic benefits after cardiomyoplasty is attributed to the damaged LDM and incomplete recovery after subtotal mobilization [5, 8]. However, there is one interesting point for consideration and investigation. All data about muscle transformation and synchronous cardiac contraction for many years without fatigue were obtained from young adult, very healthy animals with strong skeletal muscles. After that the electrical stimulation protocol was applied without change to advanced-age elderly human patients with very weak muscles due to immobilization and inactivity. However, this protocol may be too strong for elderly patients and may lead to extreme fatigue in the already weak LDM.

We hypothesized that the conditioned muscles of elderly organisms are different from the young adult in their response to electrical stimulation. We proposed that elderly skeletal muscle needs a more prolonged and careful period for transformation. The purpose of this investigation was to study the age-related skeletal muscle response to electrical stimulation, which may have a practical recommendation for patients of different ages admitted for cardiomyoplasty.

Materials and Methods

The animal studies reported in these investigations conform to the Guiding Principles Regarding the Care and Use of Animals of the American Physiological Society and to all federal laws and regulations regarding animal care approved by our institution's Animal Care Committee.

Animal preparation

Four one-year-old and four eight-year-old sheep were implanted with stimulator leads in the LDM as described below. Twenty-four hours preoperatively Amoxicillin was administered intravenously (10-15 mg/kg).

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The sheep were premedicated with diazepam (Valium; Roche, Nutley, NJ; 5mg/kg intravenously) and anesthetized with thiopental sodium (Sodium Pentothal; Abbot Hospital Products, Abbott Park, IL; 20-25 mg/kg intravenously). The sheep were then intubated, placed on a Drager ventilator, and maintained on halothane gas anesthesia (1-2% with 4.0L O₂).

In all animals, oxygen saturation level and heart rate were monitored via a pulse oximeter placed at the animal's tongue. Under sterile conditions a 7-8 cm longitudinal skin incision was made in the left mid-axillary line to expose the pedicle of the LDM. Two intramuscular electrodes were inserted into the proximal and mid portion of the left LDM perpendicular to the neurovascular pedicle. The electrodes were connected to a Myostim 7220 System (Telectronics, Englewood, CO, USA). The stimulator was implanted subcutaneously. All animals received prophylactic Amoxicillin (10-15 mg/kg) for 10 days post surgery.

Stimulation protocol

Stimulation was started with one impulse per burst, 10 Hz burst frequency, 2.5 V amplitude, and 30 contractions per minute (CPM). Every two weeks thereafter the number of pulses per burst and the burst frequency were increased. The resultant stimulation pattern after 8 weeks of stimulation was 30 CPM of 2.5V bursts containing 6 pulses at a 25 Hz burst frequency. Visible and palpable contractions of the muscle were obtained without discomfort to the animal. Stimulation was terminated at 8 weeks.

Force measurements

Contractile force (CF) measurements of the left LDM were performed before electrical stimulation, at 8 weeks of electrical stimulation and after a two week delay. For this study all animals were anesthetized with Valium (0.5 mg/kg) and Ketamine (4 mg/kg). They were intubated and artificially ventilated with oxygen/halothane. To measure CF, the front leg was connected directly in line with a precalibrated force displacement transducer (ACCU Force III, Ametec, FL, USA). All measurements were recorded on a Gould ES 1000 recorder.

Test 1

Contractile Force Test - Five consecutive measurements were taken with the leg preload set to 20 g/kg body weight at stimulator pulse amplitudes of 10 V. The pulse timing was set for six 10 Hz pulses at 60 CPM. Measurements took 5 to 6 seconds to acquire, and one minute of rest was given between measurements.

Test 2

Fatigue Test - Following Test 1, a 30 minute fatigue test was performed. The stimulator was set for 6 pulses per burst, at the fusion frequency determined for each individual animal (25-35 Hz). The amplitude was set at 10 V

and a rate of 100 CPM. Preload was 20 g/kg. CF was measured and recorded every 5 minutes.

Test 3

Fatigue-retest. After Test 1 and one-hour rest, another 30 minute fatigue test was performed. Test 3 was identical to Test 2.

Determination of the lactate dehydrogenase (LDH) isoenzyme distribution in muscle biopsy tissue

Samples of LDM (before electrical stimulation, eight weeks after beginning electrical stimulation, and after two weeks delay) were rapidly frozen in a dry ice/isopropanol bath for subsequent analysis. For the preparation of cytosolic extracts of skeletal muscle, the tissue samples were removed from storage (-70°C), weighed, placed in glass tubes (12x75 mm) and 10 volumes of ice-cold 50% glycerol buffer were added. The samples were homogenized at a setting of 5 using a Brinkman Polytron with PT7A generator until a uniform suspension was obtained (20-40 sec). The samples were then centrifuged at 30,000g for 20 minutes. The floating lipid layer was removed and the supernatant was aspirated into a clean tube and stored at -40°C. Protein concentrations were determined fluorometrically using dilutions of the homogenate supernatants and bovine serum albumin as standard.

The isoenzymes of LDH were separated on ion-exchange mini-columns. Homogenate supernatants of sheep muscle were diluted with 20 mmol/L BIS TRIS (pH 6.3) buffer to obtain a total LDH activity of < 3 U/ml. The BIS TRIS buffer was prepared by dissolving 10.46 g BIS TRIS, 0.1752 ml β-mercaptoethanol, and 2.08 g EDTA-Na₄•2H₂O in water, adjusting the pH to 6.3 with HCl, and adding water to 500 ml total volume, and 1.0 M NaCl was prepared by dissolving 14.61 g NaCl in water to a total volume of 250 ml.

Aliquots of the homogenate (0.7 ml) were applied to DE 52 columns (0.7x6 cm) that had been equilibrated with BIS TRIS buffer. Then, 4 ml (2x2 ml) of the buffer was eluted through the column followed by 4 ml 0.06 M NaCl buffer and 4 ml 0.15 M NaCl buffer. The 0 M NaCl buffer eluted LDH-5, 0.06 M NaCl buffer eluted LDH4+3, and 0.15 M NaCl buffer eluted LDH2+1. LDH activity was determined at 30°C in 1.0 ml total volume by measuring the reduction of NADH at 340 nm with pyruvate as substrate.

Transmission electron microscopy

Biopsies of LDM (approximately 3x4 mm²) (at the same times listed for LDH determination) were placed into Karnovsky's fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M Sodium Cacodylate buffer, pH 7.2) and then minced into smaller (1-2 mm²) pieces. The biopsies were post-fixed in a 1% Osmium Tetroxide, dehy-

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drated through a series of graded alcohols and acetone, and embedded in Spurr resin.

Preliminary thick (1 μm) sections were cut and stained with 0.1% Toluidine blue. Longitudinal areas of muscle were selected for ultra-thin sectioning; thin sectioning (60-90 nm) was done with a Reichert Ultra-Cut microtome. The thin sections were stained with 5% Uranyl Acetate and Reynold's Lead stain (3.5% lead citrate, 2% lead nitrate). Examination and photography of the thin sections were done with a Philips 400T Transmission Electron microscope (TEM) at an accelerating voltage of 60 kV using Kodak SO-163 image film.

Five electron micrographs of adjacent areas in each of 2 regions were taken on each biopsy at a magnification of 6000x. A 2800x magnification of the two regions was also included as an overview. Photographic enlargements ($8 \times 10 \text{ in}^2$) were made of each micrograph with a final magnification of 16740x and 7812x, respectively.

Determination of mitochondrial area

Ten random TEM micrograph photos were chosen from each biopsy sample. Two photocopies of each photo were made and weighed (original weight or OW) after cutting off the waste edges. Areas of large mitochondria were removed from the copies with scissors; areas of smaller mitochondria were burned off with electric cauter. After all areas of mitochondria were excised, the copies were again weighed (cut weight or CW). The percentage of mitochondrial area was calculated using the following formula: $[(\text{OW}-\text{CW})/\text{OW}] \times 100$. For our data, the mean value of each photo was calculated from two different copies, and the mean value of one biopsy sample was the average of the ten photos. To evaluate the reproducibility of this method, ten copies of the same picture were cut and weighed. The correlation was 94%.

Determination of the number of nuclei and fibers

A sample of approximately 3x4 mm was placed in 10% formaldehyde solution for analysis by light microscopy. The samples then were sent to hospital's pathology department to be embedded and cut. Transverse sections were stained with methylene blue and basic fuchsin stains. Multiple slides were made for each sample. Sections were examined under light microscopy, and the number of nuclei and number of fibers were calculated per area of muscle cross section. Results are given as number per millimeters squared.

Data analysis

Statistical analysis of the results was made by Student's T-test. Results are expressed as mean \pm standard error of the mean (SEM). Data with a p value of < 0.05 were considered statistically different.

Results

Untrained skeletal muscle

Contractile force (fig. 1)

Contractile force of untrained LDM in one-year old sheep was 1766 ± 48 g with preload at 20 g/kg and amplitude of 10 V. Contractile force of untrained LDM in eight-year old sheep was less: 1533 ± 74 g ($p < 0.05$).

Fatigue resistance

The untrained LDM of 1 year-old sheep lost $36 \pm 4\%$ CF during the first 5 minutes of testing. After 30 minutes $43 \pm 5\%$ CF had been lost (1006 ± 53 g) The untrained LDM of 8 year-old sheep lost $40 \pm 7\%$ CF during the first 5 minutes of testing. After 30 minutes $46 \pm 9\%$ CF had been lost (833 ± 61 g). Difference between contractile force after testing was statistically significant ($p < 0.05$) (fig. 2).

Lactate dehydrogenase isoenzyme distribution

Total LDH units per milligram protein in one year old sheep was 9.0 ± 0.8 ; LDH-5 fraction was $91 \pm 3\%$, and LDH-1+2 fractions were $1.2 \pm 0.3\%$. Total LDH units per milligram protein in 8 year old sheep was 8.3 ± 0.6 ;

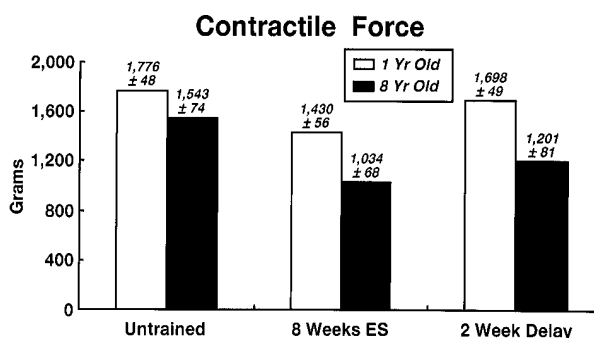


Figure 1. Contractile force of untrained latissimus dorsi muscle after 8 weeks of electrical stimulation and 2 weeks after cessation of electrical stimulation (delay). Test stimulus conditions: 20 g/kg preload, six 10 Hz pulses per burst, 10 V amplitude.

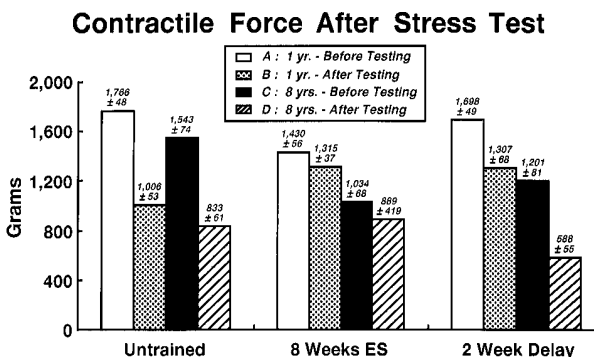


Figure 2. Contractile force after a 30 minute stress test. The stimulator was set for six pulses per burst, fusion frequency (25-35 Hz - varies with individual animal), 10 V amplitude, 20 g/kg preload, and 10 contractions per minute.

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LDH-5 fraction was $92\pm 4\%$, and LDH-1+2 fractions were $1.0\pm 0.6\%$ (table 1).

Percent of mitochondria area

The percent area occupied by mitochondria in untrained LDM was $5.2\pm 2.0\%$ in one year old sheep and $4.2\pm 0.7\%$ in 8 year old sheep (table 1).

Percent area occupied by muscle fibers

The percent area occupied by muscle fibers in untrained LDM was $82\pm 3\%$ in one year old sheep, and $73\pm 4\%$ in 8 year old sheep. There were statistically significant differences between 8-year-old sheep and one year old sheep ($p < 0.05$).

Number of nuclei and fibers per millimeters squared

In one year old sheep the number of nuclei per millimeters squared in unconditioned LDM was 517 ± 55 , the number of fibers was 320 ± 45 , and the ratio of nuclei to fibers was 1.60. In 8-year-old sheep the number of nuclei was 431 ± 63 , the number of fibers was 306 ± 33 , and the ratio of nuclei to fibers was 1.43 (table 1).

Skeletal muscle after 8 weeks electrical stimulation

Contractile force

In one year old sheep contractile force decreased to 1430 ± 56 g ($81\pm 7\%$ from control). In eight year old sheep contractile force decreased to 1034 ± 68 g ($67\pm 4\%$ from control). There were statistically significant differences ($p < 0.05$) between old (8 year old sheep) LDM and the LDM of one-year-old sheep. Very important to note that compared to one-year old sheep (1430 ± 56 g) contractile force of the LDM of 8 year old sheep (1034 ± 68 g) was 28% less (fig 1).

Fatigue resistance

After 30 minutes of testing, the LDM of 1 year old sheep also lost only $8\pm 3\%$ of initial contractile force (from 1430 ± 56 g to 1315 ± 37 g). No fatigue was evident during the first 10 minutes of testing. The LDM of 8 year old sheep had greater fatigue than younger muscle: LDM lost $14\pm 5\%$ of its initial contractile force (from 1034 ± 68 g to 889 ± 41 g). The decrease in contractile force began two minutes after starting the fatigue test (fig. 2). There were no statistically significant differences ($p > 0.05$)

between the two groups of animals, but there was a tendency for older muscle to be more fatigable during the testing. However, if we compare the final contractile force after testing (1315 ± 37 g for one year old sheep and 889 ± 41 g for 8 year old sheep) differences are significant ($p < 0.05$). If we calculate the final contractile force of one year old sheep as 100%, the final contractile force of 8 year old sheep will be only 67%.

Lactate dehydrogenase isoenzyme distribution

In one year old sheep after 8 weeks of electrical stimulation the LDH-5 fraction decreased from $91\pm 3\%$ to $74\pm 8\%$ and LDH-1+2 fractions increased from $1.2\pm 0.3\%$ to $8.4\pm 0.4\%$. In 8 year old sheep LDH-5 fraction decreased from $92\pm 4\%$ to $84\pm 3\%$ and LDH 1+2 fractions increased from $1.0\pm 0.6\%$ to $3.2\pm 1.1\%$ (table 1). There was a statistically significant ($p < 0.05$) increase in the LDH-1+2 fractions in one-year-old sheep compared with the older sheep.

Percent of mitochondrial area

The percent area occupied by mitochondria after 8 weeks of electrical stimulation increased in one-year old sheep from $5.2\pm 2.0\%$ to $6.9\pm 1.3\%$. In eight year old sheep the area increased less than in the younger animals (from $4.2\pm 0.7\%$ to $5.3\pm 0.8\%$) (table 1) but was statistically insignificant ($p > 0.05$).

Percent area occupied by muscle fibers

After 8 weeks of electrical stimulation the percent area occupied by muscle fibers decreased in one year old sheep from $82\pm 3\%$ to $74\pm 5\%$, and in eight year old sheep from $74\pm 3\%$ to $65\pm 2\%$. Eight-year-old sheep initially had less area occupied by muscle fibers compared with younger animals ($p < 0.05$). After electrical stimulation the area of muscle fibers decreased in older sheep, which was also statistically significant ($p < 0.05$) compared with one year old sheep. After electrical stimulation at least $35\pm 2\%$ of the muscle of older animals was occupied by connective tissue and fat, which did not participate in the muscle contraction.

Number of nuclei and fibers per millimeters squared

After electrical stimulation the number of nuclei in one year old sheep increased from 517 ± 55 to 994 ± 52 ,

Table 1

	Unconditioned Muscle		Muscle After 8 Weeks ES		Muscle After 2 Weeks Delay	
	1 year old	8 years old	1 year old	8 years old	1 year old	8 years old
LDH-5 fraction (%)	91±3	92±4	74±8	84±3	83±3	90±5
LDH-1+2 fraction (%)	1.2±0.3	1.0±0.6	8.4±0.4	3.2±1.1	4.6±0.3	2.5±0.4
Mitochondrial area (%)	5.2±2.0	4.2±0.7	6.9±1.3	5.3±0.8	5.1±1.3	4.3±1.0
Number of nuclei	517±55	431±63	994±52	618±91	576±47	483±37
Number of fibers	320±45	306±33	560±47	391±43	351±41	310±42

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and in eight year old sheep from 431 ± 63 to 618 ± 91 . There were statistically significant differences ($p < 0.05$) between the eight-year-old sheep and the younger animals. The number of fibers increased (i.e. the size of fiber began to get smaller) in one year old sheep from 320 ± 45 to 560 ± 47 , and in eight year old sheep from 306 ± 33 to 391 ± 43 per mm^2 (table 1). Again there were statistically significant ($p < 0.05$) differences between the older and younger animals. The ratio of nuclei to fibers increased in the one year old sheep (1.77 vs 1.60) and in eight year old sheep (1.58 vs 1.43).

Skeletal muscle after two week delay (without electrical stimulation)

Contractile force

After 2 weeks delay the contractile force of the LDM of young sheep increased from 1430 ± 56 g to 1698 ± 49 g ($96\pm 4\%$ to control). The contractile force was considerably stronger than immediately after completing electrical stimulation training ($p < 0.05$) and practically restored its potential as before electrical stimulation ($p > 0.05$). Contractile force of the LDM of 8 year old sheep increased only to 1201 ± 81 g ($p > 0.05$ compared to 8 weeks electrical stimulation, but $p < 0.05$ compared to control) (fig. 1).

Fatigue resistance

After 30 minutes of testing the LDM of one-year-old sheep lost $33\pm 6\%$ contractile force (from 1698 ± 81 g to 1307 ± 68). After the delay eight-year-old sheep lost $51\pm 4\%$ of initial contractile force (from 1201 ± 81 g to 588 ± 55 g) (fig. 2). There were statistically significant ($p < 0.05$) differences in fatigue resistance between young and old sheep after the delay.

Lactate dehydrogenase isoenzyme distribution

As expected, after two weeks delay the LDH fractions changed back to baseline in adult sheep. In one-year old sheep LDH-5 fraction increased to $83\pm 3\%$ (from $74\pm 8\%$) and in eight-year-old sheep to $90\pm 5\%$ (from $84\pm 3\%$). In one-year-old sheep LDH 1+2 fractions decreased to $4.6\pm 0.3\%$ (from $8.4\pm 0.4\%$ after electrical stimulation), and to $2.5\pm 0.4\%$ in eight-year-old sheep (from 3.2 ± 1.1 after electrical stimulation) ($p < 0.05$) (table 1).

Percent of mitochondria area

The percent area occupied by mitochondria after a two week delay decreased to $5.1\pm 1.3\%$ in one year old sheep (before electrical stimulation $5.1\pm 1.3\%$), and to $4.3\pm 1.0\%$ in eight-year-old sheep (before electrical stimulation $4.3\pm 0.7\%$) (table 1).

Percent area occupied by muscle fibers

In one year old sheep the percent area increased from $74\pm 3\%$ to $78\pm 4\%$, but continued to be less than before stimulation ($82\pm 3\%$). In eight year old sheep the area oc-

cupied by muscle fibers continue to be decreased as after 8 weeks of electrical stimulation ($63\pm 8\%$ vs $65\pm 2\%$).

Number of nuclei and fibers per millimeter squared

In the one-year-old sheep after a two week delay, the number of nuclei (576 ± 47 vs 994 ± 52) and the number of fibers reverted to baseline values (351 ± 41 vs 560 ± 47). The ratio of nuclei to fibers was similar (1.61 vs 1.60). Also in the eight-year-old sheep the number of nuclei (483 ± 37 vs 618 ± 91) and number of fibers (310 ± 42 vs 391 ± 43) reverted to baseline. The ratio of nuclei to fibers was 1.55 (before stimulation 1.58) (table 1).

Discussion

In our preliminary investigations [3] we showed that the LDM in newborn lambs had more plasticity than one year old sheep. For clinical use it is important to know the differences between young adult skeletal muscle and older adult skeletal muscle. If the difference is dramatic, it may be necessary to change the electrical stimulation protocol for elderly patients undergoing cardiomyoplasty. Our investigation showed that there was no statistically significant ($p > 0.05$) differences in untrained skeletal muscle between one-year-old and eight-year-old sheep in fatigue resistance (after 30 minutes of intensive stress testing), LDH isoenzyme distribution, the percent of mitochondria area, and the number of nuclei and fibers per millimeter squared. However, the percent area occupied by muscle fibers was less in eight-year-old sheep compared with the one-year-old sheep. With age, muscle fibers are replaced by connective and fat tissue, and muscle transformation using electrical stimulation will be more difficult in an elderly group of animals. The first evidence that older skeletal muscle did not adapt with electrical stimulation as in younger muscle was the considerable decrease in contractile force: in eight-year-old sheep contractile force decreased to $67\pm 4\%$ compared with $81\pm 7\%$ in one-year-old sheep. The muscle must pay for the acquisition of fatigue resistance with a decrease in force, but such a considerable loss of contractile force in the older muscle is a predictor of a future insufficient contribution to cardiac assist. However, after 8 weeks of electrical stimulation training the older LDM began to be more fatigue resistant: during a fatigue test it lost only $14\pm 5\%$ of pre-testing contractile force. At first glance, this is not significant when compared with younger muscle which lost $8\pm 2\%$ of initial force. But after completion of the stress test, the contractile force of the older LDM was only 53% compared with pretraining values. In the younger muscle the CF was 73%. There was no difference in the percent of mitochondrial area – it increased in all groups.

For adequate muscle transformation from quick-twitch to slow twitch, it is very important to alter the amounts of the LDH fractions. The LDH-1+2 fraction increased and the LDH-5 decreased considerably less in older LDM than in the LDM of one-year-old sheep. Therefore the

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process of transformation for this variant of biochemical metabolism in the older muscle was slower than in young muscle. There is also other evidence: the number of nuclei per millimeter squared in eight-year-old sheep was considerably less than in one-year-old sheep. With complete transformation the size of the muscle fiber decreases and the number per millimeter squared increases. This process was considerably less in the older muscle (391 ± 43 vs 560 ± 47). In the older LDM after electrical stimulation, at least 35 % of the muscle was occupied by connective tissue and fat which does not participate in muscle contraction. Accordingly, the older LDM did not complete the transformation after 8 weeks of training and thus will perform less cardiac assistance than younger LDM. For older LDM, it may be necessary to prolong the training protocol using a more cautious regimen, and after training allow this muscle to have a longer period of rest between contractions.

We received confirmation that the older muscle did not complete its transformation with our investigation after a two week delay. During this period the LDM received no stimulation. Contractile force after stress testing, LDH fractions, the percentage of mitochondria, and the number of nuclei and fibers per millimeter squared returned to baseline values. There was no change in the percent area occupied by muscle fibers. This data allows us to conclude that a conventional electrical stimulation protocol applied to older skeletal muscle will not convert the LDM completely to a fatigue resistant state, and that after a two week period without stimulation, all acquired properties revert to preconditioned values.

Conclusions

1. It is a mistake to apply the electrical stimulation protocol designed for healthy, adult animals to very sick, advanced-age human patients. This disparity may be one reason that poor hemodynamic results are received in follow-up after dynamic cardiomyoplasty.
2. Elderly skeletal muscle does not convert to a fatigue resistant state as completely as young adult skeletal muscle during conventional 8 week ES protocol, and cannot perform the work that is expected from it after cardiomyoplasty. It is necessary to modify and extend the ES protocol for elderly patients.

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